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The most accurate and sensitive methods available for the estimation of reducing sugars are those which employ oxidizing reagents. It is, of course, generally recognized that such methods are not specific for sugars, and numerous techniques have been proposed for the removal of interfering substances which are commonly present in solutions or extracts of biological origin. It appears to be less generally appreciated that certain procedures employed in connection with carbohydrate analyses may in themselves introduce interfering substances.

The present report describes the effects of a number of compounds, chiefly salts of organic and inorganic acids, on the determination of reducing sugars by copper and by ferricyanide reagents which have been widely used for microanalysis of biological materials.

METHODS

Copper reagent.—The copper-iodometric reagent #50 of Shaffer and Somogyi (1933) has been employed. For the sake of completeness details of its preparation and use are included.

Fifty gm. of sodium carbonate and 50 gm. of Rochelle salt are dissolved in about 1,200 ml. of distilled water. One hundred and fifty ml. of 0.4 M copper sulfate are then added by pipet or funnel extending below the surface of the liquid, the solution being stirred. Forty gm. of sodium bicarbonate and 2 gm. of potassium iodide are then dissolved in the solution, and 50 ml. of 0.8 N potassium iodate (1.427 gm. KIO_3) are added. The solution is made to 2 l.

The sugar determination is carried out as follows. Ten ml. of test solution are mixed with 10 ml. of reagent in a 25 × 200 mm. Pyrex test tube which is then closed by a rubber stopper fitted with a Bunsen valve. The tube is immersed in a boiling-water bath to such a depth that the water is a few centimeters higher than the solution level. After 15 minutes (measured to within ± 5 seconds) the tube

is transferred to a bath of cold water for a few minutes. Two ml. of a solution containing 2.5 percent potassium iodide and 2.5 percent potassium oxalate are then added, followed by 2 ml. of 5 N sulfuric acid; the acid should be added rapidly while holding the test tube in an inclined position to avoid loss of solution through undue effervescence. After the stopper is replaced, the tube is shaken to effect solution of the precipitated cuprous oxide, and allowed to stand for 5 minutes. The liberated iodine is then titrated with 0.02 N sodium thiosulfate in the presence of starch.

Each day a blank determination is made in exactly the same way, with water instead of sugar solution. The difference between the titration values of the blank and of the test solution is the volume of thiosulfate equivalent to the sugar; the value of the latter can be calculated from a curve or table constructed from analyses of pure sugars. Such a curve for glucose is given by Heinze and Murneek (1940); our results agree very closely with theirs.

With the reagent as above described, amounts of glucose up to 4.4 mg. in 10 ml. may be determined. With careful measurement of the reagent and test solutions, and titration with a buret calibrated to 0.05 ml., duplicate analyses of pure solutions usually agreed within 0.02 ml. which is equivalent to an error of about 0.4 percent in the determination of 2 mg. of glucose. Greater differences were encountered in the presence of some of the substances tested.

Ferricyanide reagent.—The ferricyanide reagent, patterned after the Hanes (1929) modification of that of Hagedorn and Norman Jensen (1923), is a solution 0.025 M in potassium ferricyanide and 0.1 M in sodium carbonate. The reaction is carried out with 10 ml. of reagent and 10 ml. of sugar solution in exactly the same manner as described for the copper reagent. After cooling the tubes there are added 5 ml. of a solution containing 25 gm. KI, 50 gm. $ZnSO_4 \cdot 7H_2O$, and 250 gm. NaCl per liter, and then 3 ml. of 1 N acetic acid. The liberated iodine is titrated with 0.025 N $Na_2S_2O_3$. Blanks are run and the calculations made as with the copper reagent.

With this procedure a linear relation obtains between the titration values and the amount of reducing sugar present (see also Hulme and Narain, 1931; Heinze and Murneek, 1940); up to about 7 mg. of glucose can be determined. The difference between duplicate determinations of a pure sugar solution is usually not greater than 0.01 ml. $Na_2S_2O_3$, which is equivalent to an error of about 0.4 percent in the analysis of 2 mg. of glucose.

In a number of experiments the iodometric determinations were checked by estimation of the ferrocyanide produced, according to

the method of Hassid (1937). After cooling the reaction mixture, 5 ml. of 5 N sulfuric acid and 0.5 ml. of 0.1 percent aqueous Setopaline C are added and the ferrocyanide titrated with 0.02 N ceric ammonium nitrate in 1 N H_2SO_4. Concordant results were given by the two methods except in the presence of oxalate, where cerimetric estimation is unsuitable.

RESULTS AND DISCUSSION

Figures 1 to 8 show the effects of the incorporation in the sugar solution of various substances to give the indicated concentrations. (Concentrations of the test substances in the reaction mixtures are of course only one-half as great.) Results are expressed as percentage deviation from the simultaneously measured value for pure sugar. These results are calculated on the basis of blank determinations using water; in a few instances blanks were determined also with solutions of the test substances but inasmuch as, on the one hand, these differed only relatively slightly from the water blanks and, on the other, the primary purpose of this investigation was to ascertain the limits of accuracy of the analytical methods as routinely performed, no correction has been made for this effect.

Two mg. of glucose (National Bureau of Standards dextrose) were used in all experiments except where otherwise specified. The substances tested were of C.P. or reagent quality with the exception of potassium acetate, sodium benzoate, and ethyl alcohol, which were of U.S.P. grade.

Sodium chloride, potassium chloride, sodium fluoride (fig. 1).— It is well known that in the presence of high concentrations of chloride the copper reduction equivalent is diminished. This effect is attributed to the greater ease of reoxidation of the cuprous oxide which is held in solution by the halide (Shaffer and Somogyi, 1933).

Wood (1935) stated that in the presence of KCl, the rate of oxidation of glucose by ferricyanide was diminished but that the final amount of oxidant reduced was increased. Inasmuch as the oxidation reaction in the absence of added salt is nearly complete in 7 to 8 minutes, our finding of increased oxidation (after 15 minutes) in presence of halides appears to be in agreement with Wood's observation.

Concentrations of chloride sufficient to effect an appreciable error may be introduced by certain analytical techniques, e.g., the hydrolysis of disaccharides and polysaccharides by strong hydrochloric acid. Significant error is occasioned also by the amounts of sodium fluoride sometimes added for preservation of biological extracts.

Sodium sulfate, potassium sulfate (fig. 1).—According to Somogyi (1937), sulfate has a manifold effect upon the copper reduction. On the one hand it tends to increase the reduction equivalents by depressing the solubility of oxygen in the reaction mixture, and by decreasing the alkalinity of the solution through suppression of the ionization of the carbonate; on the other hand it decreases the rate of reduction so that in a given time the reaction is nearer completion in the absence of sulfate than in its presence. Under our conditions, the last effect appears to predominate. The effect of potassium sulfate (not shown in fig. 1) is identical with that of sodium sulfate.
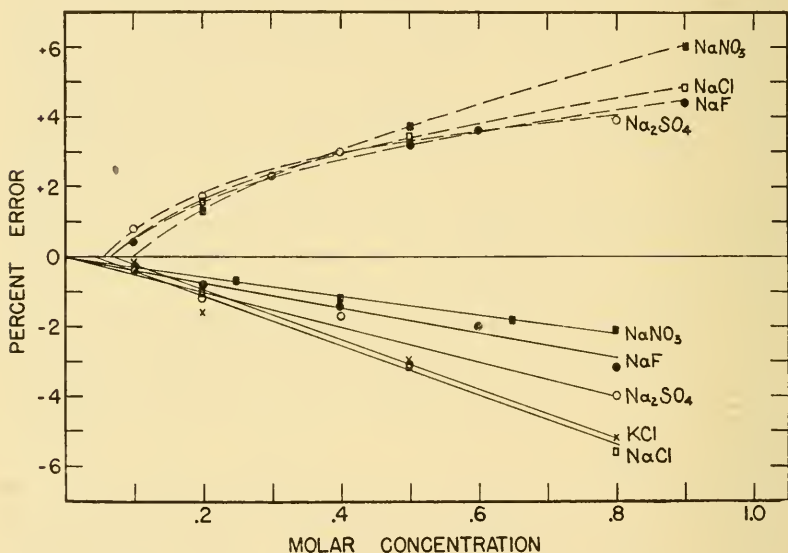

Fig. 1.—Effects of sodium fluoride, sodium chloride, potassium chloride, sodium nitrate, and sodium sulfate on estimation of glucose by copper (———) and ferricyanide (– – –) reagents.

The effect of alkali sulfates upon oxidation by the ferricyanide reagent is practically identical with that of the halides.

Sulfate is sometimes used to precipitate lead from sugar solutions which have been clarified by this metal. A large excess of sulfate should be avoided. Sulfate is introduced into sugar solutions also by the use of sulfuric acid for hydrolysis of carbohydrates.

Sodium nitrate (fig. 1).—This salt produces effects on both reagents similar to those given by halide and sulfate.

Calcium chloride, magnesium chloride, magnesium sulfate (fig. 2).— These salts cause a marked diminution of the copper reduction, presumably owing chiefly to precipitation of carbonate and consequent

decrease of the alkalinity of the reaction mixture. On the basis of the findings with NaCl and Na_2SO_4, only a small portion of the effect would appear to be due to the amounts of these salts formed in the reaction. Carbonate is precipitated from the reagent at room temperature by an equal volume of solutions 0.01 M or greater in calcium. Magnesium, in the concentrations tested, produces a pre-
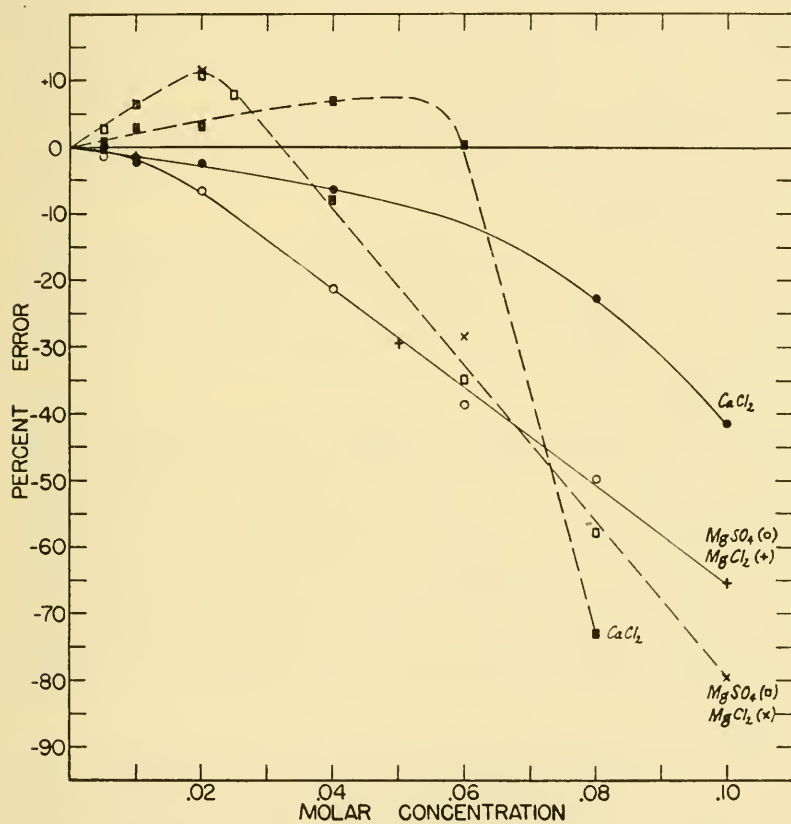

Fig. 2.—Effects of calcium chloride, magnesium chloride, and magnesium sulfate on estimation of glucose by copper (———) and ferricyanide (– – –) reagents.

cipitate only on heating. On a molar basis, magnesium has approximately twice the effect of calcium. The explanation of this result is not clear; it seems likely that a basic magnesium carbonate is formed, possibly a double salt with sodium carbonate.

Interference of calcium in sugar determination by Fehling's solution was observed by Eynon and Lane (1923) and confirmed by

Cook and McAllep (1928). As Fehling's solution contains no carbonate the Ca presumably reacts with NaOH. Magnesium salts were found not to exhibit a comparable interference, however (Eynon and Lane). This circumstance underlines the inadvisability of generalizing the results obtained with a particular copper reagent.

The curves for the ferricyanide reagent exhibit sharp maxima, the lower concentrations of Ca and Mg producing an increase in the amount of reduction while higher concentrations cause a marked decrease. Hence two opposing effects seem to occur in the presence of these metals. Much less pronounced inflections are indicated at the corresponding concentrations in the copper reagent curves. Solutions of $Mg^{++} > 0.02$ M and of $Ca^{++} > 0.06$ M cause precipitation of carbonate when mixed with equal volumes of the ferricyanide reagent at room temperature.

Calcium is sometimes deliberately introduced into sugar solutions, e.g., in the procedure of Kerstan (1934) in which HCl used for hydrolysis of maltose is neutralized by a mixture of NaOH and $Ca(OH)_2$ in order to prevent the inhibitory effect of NaCl upon yeast fermentation.

Ammonium chloride, ammonium sulfate (fig. 3).—Ammonium salts produce an appreciable effect in rather low concentration. Cupric-ammonia coordination compounds are formed and no cuprous oxide precipitates from solutions which are more than 0.04 M in NH_4^+. Eynon and Lane (1923) also reported interference by low concentrations of NH_4Cl in determinations with Fehling's solution, and Folin and Svedberg (1926) described a copper reagent containing a high concentration of $(NH_4)_2SO_4$ which did not react with reducing sugar.

The results with the ferricyanide reagent resemble those produced by $CaCl_2$.

Sodium hydroxide, sodium carbonate, sulfuric acid (figs. 4, 5).—The influence of alkalinity upon reagent #50 was studied by Shaffer and Somogyi (1933), who found that the higher the alkalinity the faster the sugar oxidation but the lower the final amount of copper reduced; the latter finding is confirmed by our results with NaOH. Added Na_2CO_3, up to a concentration of 0.175 M in the sugar solution, has no influence owing to the buffer capacity of the carbonate + bicarbonate content of the reagent; with higher concentrations impermanent end points were obtained in the thiosulfate titration. The low results obtained on addition of sulfuric acid are presumably due to retardation of the reaction.

Pickett (1940) found that the pH of reagent #50 (about 9.2)

was appreciably altered on mixing with equal volumes of various unclarified plant juices.

The influence of alkalinity on sugar oxidation by ferricyanide has been reported to be similar to that found in oxidation by copper (Van Slyke and Hawkins, 1928; Wood, 1935). Our results with ferricyanide differ considerably from those with copper, however. With
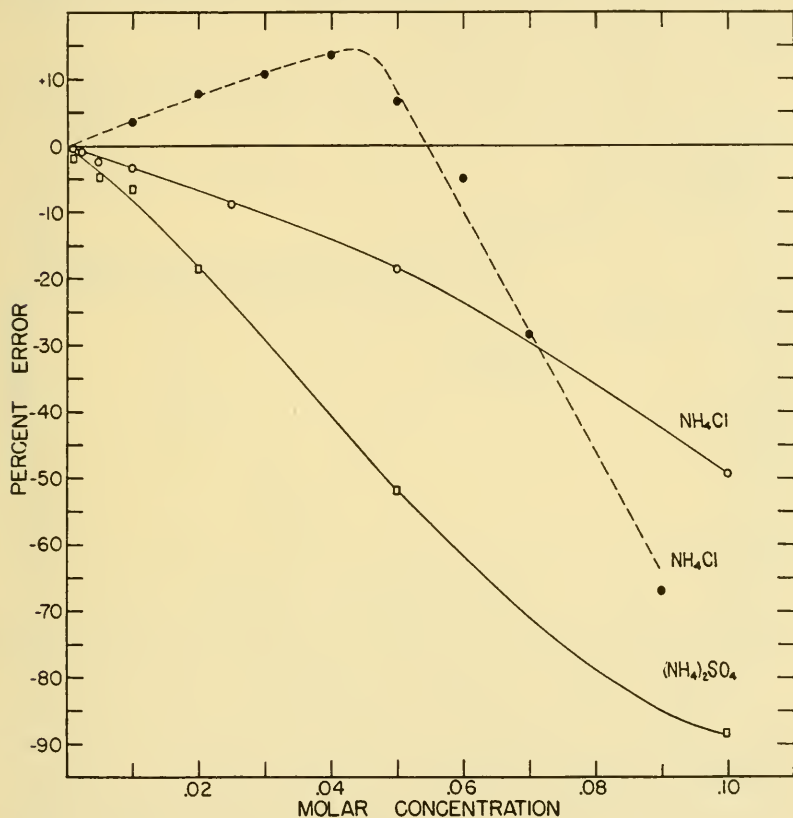

Fig. 3.—Effects of ammonium chloride and ammonium sulfate on estimation of glucose by copper (———) and ferricyanide (– – –) reagents.

increasing concentrations of NaOH the amount of reduction in 15 minutes first decreases, then passes through a minimum and finally increases. Conversely, with increasing concentrations of sulfuric acid the amount of reduction decreases initially, attains a maximum, and then diminishes rapidly.

Sodium carbonate produces only an increase in the amount of ferricyanide reduced. Similar findings have been reported by Englis and

Becker (1939). In using Na_2CO_3 for deleading extracts clarified with lead acetate an excess should be avoided if reducing sugars are to be determined by ferricyanide.

The acid results appear to conform to the pattern of the family of curves published by Van Slyke and Hawkins, but the results with alkali do not seem explicable on the basis of the previous findings. A family of time curves for various degrees of alkalinity would be required to clarify the situation.
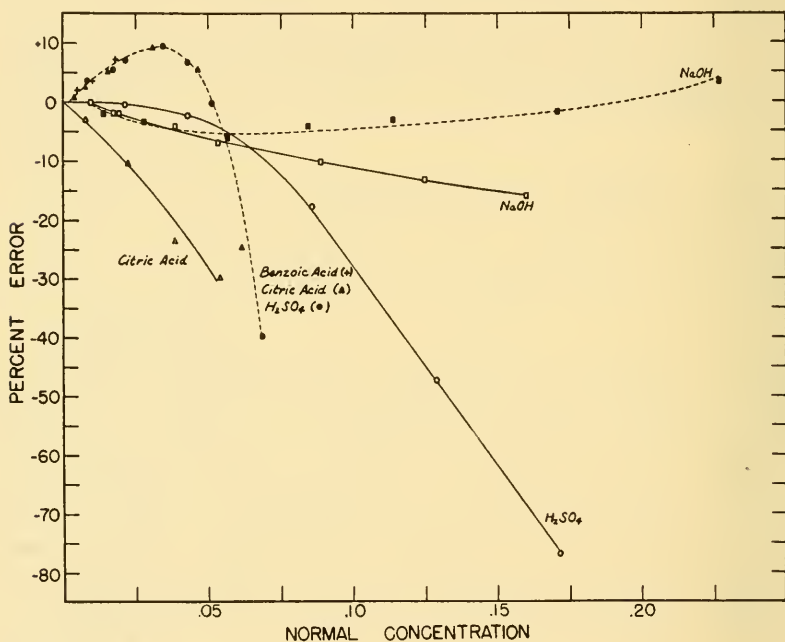

Fig. 4.—Effects of sulfuric acid, citric acid, benzoic acid, and sodium hydroxide on estimation of glucose by copper (———) and ferricyanide (– – –) reagents.

Monosodium phosphate, dipotassium phosphate, tripotassium phosphate (fig. 5).—The effects of NaH_2PO_4 on both reagents resemble those produced by H_2SO_4 and doubtless are due, in part at least, to the increased hydrogen-ion concentration. A precipitate is formed in the copper reagent on mixing with an equal volume of 0.2 M NaH_2PO_4.

Dipotassium phosphate (or disodium phosphate), which tends to depress the pH of the reagents, also gives results which might be expected from such an effect. Stable end points cannot be obtained by the iodometric method under the conditions employed,

if the concentration of K_2HPO_4 equals 0.2 M in the copper reaction mixture or 0.1 M in the ferricyanide reaction mixture.

The inhibiting effect of disodium phosphate on the oxidation of glucose has been observed previously (Fischl, 1933; Strepkov, 1936; Englis and Becker, 1939) and has been utilized in the construction of reagents selective for fructose, inasmuch as the oxidation of this sugar is affected to a much smaller degree. Decreased oxidation of glucose by a copper reagent in the presence of KH_2PO_4 was noted by Visscher (1926).

Tripotassium phosphate might be expected to exert an influence by virtue of its alkalinizing action, and indeed the results obtained
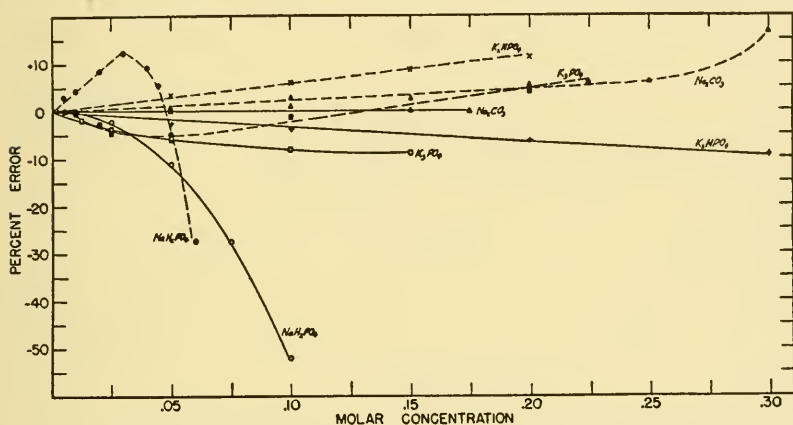

Fig. 5.—Effects of monosodium phosphate, dipotassium phosphate, tripotassium phosphate, and sodium carbonate on estimation of glucose by copper (———) and ferricyanide (– – –) reagents.

with both sugar reagents resemble those produced by NaOH. It is apparent from the curves, however, that some other effect must be operative also. Impermanent end points are obtained in titration of copper and ferricyanide reaction mixtures which are at least 0.10 and 0.125 M in K_3PO_4, respectively.

Potassium acetate (fig. 6).—Acetates of lead are very widely employed in clarification of plant extracts prior to analysis. The added acetate remains in the solution after removal of the lead. With the copper reagent no error is introduced by potassium acetate in concentrations less than 0.3 M; in the presence of higher concentrations the determination becomes unsatisfactory owing to impermanence of the end point.

Although the ferricyanide reagent gives somewhat high results at all concentrations of acetate, the error introduced by the maximal

amounts of lead acetate customarily used would be less than 0.5 percent.

Potassium oxalate (fig. 6).—Oxalate is commonly employed as an anticoagulant for blood. The amount customarily added is such as to furnish a maximal concentration of about 0.01 M which causes an error of only 0.5 percent with the copper reagent and less than this with the ferricyanide reagent. Occasional directions, however, call for the use of 2 to 5 times this amount of oxalate which is sufficient to produce an appreciable error with either reagent.

Oxalate is frequently used also for deleading plant extracts clarified by lead acetate. Care should be taken that only a small excess of oxalate is added.
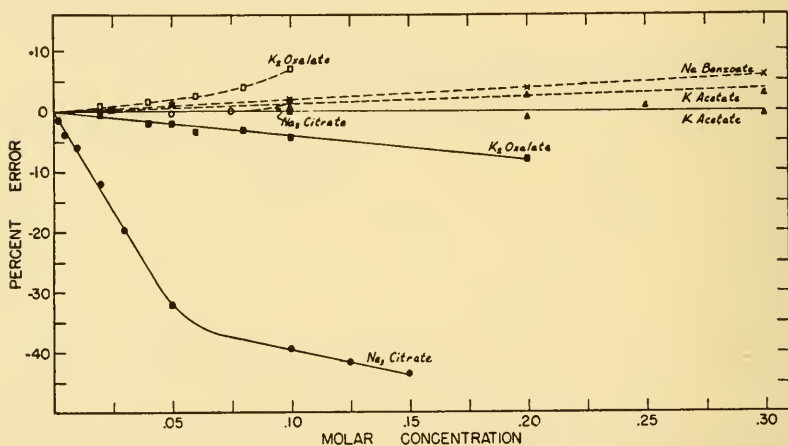

FIG. 6.—Effects of potassium acetate, sodium benzoate, sodium citrate, and potassium oxalate on estimation of glucose by copper (————) and ferricyanide (– – –) reagents.

The action of oxalate on copper reagents has been described by Shaffer and Hartmann (1921).

Sodium citrate, citric acid (figs. 4, 6).—The copper reagent is more sensitive toward citrate than to any of the other substances tested. The free acid apparently combines the specific effect of the citrate ion and the general hydrogen-ion effect.

The ferricyanide reaction is very much less sensitive to the citrate ion. This is shown clearly also by the curve for citric acid which coincides with that of sulfuric acid. Sodium citrate in concentrations greater than 0.1 M causes impermanent end points; a similar observation was made by Hulme and Narain (1931), who attributed it to interference with the precipitation of ferrocyanide as potassium

zinc ferrocyanide. The interference could be avoided by adding a sufficiently large amount of $ZnSO_4$.

In determinations with the copper reagent serious error may attend the use of citrate as an anticoagulant for blood, or the use of citric acid for hydrolysis of disaccharides. Conceivably, some of the findings in the literature of higher reducing sugar content of blood by ferri-cyanide determination than by copper reagents may be at least partially attributable to the use of citrate.

Sodium benzoate, benzoic acid.—These substances were tested because, owing to their antiseptic action in low concentrations, they are sometimes employed as preservatives for sugar solutions and extracts. With the copper reagent no significant error was found in the oxidation of glucose solutions containing 0.016 M benzoic
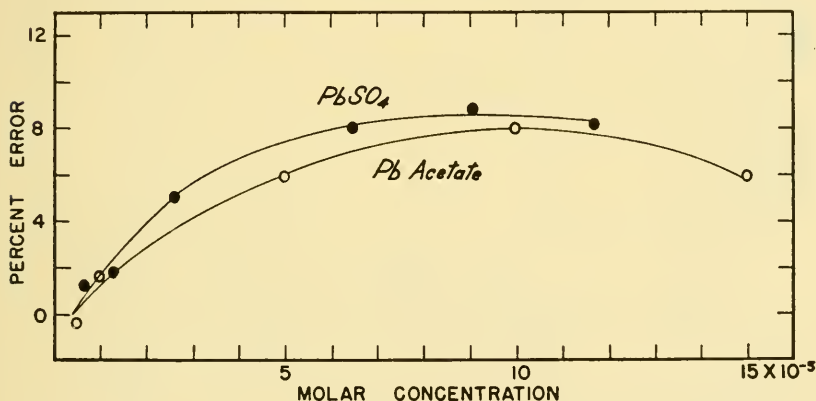

FIG. 7.—Effects of lead sulfate and normal lead acetate on estimation of glucose by ferricyanide reagent. (Solubility of $PbSO_4$ taken as 13×10^{-5}M at 20°C.).

acid (nearly a saturated solution) or 0.1 M sodium benzoate (from which a considerable precipitate of benzoic acid is produced by acidification).

The ferricyanide reaction, however, is much more sensitive toward both substances (figs. 4, 6).

Lead sulfate, lead oxalate, lead carbonate, lead sulfide, lead phosphate.—These salts were prepared by mixing solutions of normal lead acetate with excess of the alkali salts of the appropriate anions. The precipitates were filtered and washed until the wash water gave no test for the anions. Saturated solutions were prepared by shaking in water at room temperature on a mechanical shaker for several hours over a period of some days, and filtering the suspensions.

With glucose solutions containing these salts in concentrations equal to eight-tenths of saturation, the following percentage errors were found by means of the copper reagent: $PbSO_4$, 0.0; PbC_2O_4, −0.2; $PbCO_3$, −0.2; PbS, −0.2; $Pb_3(PO_4)_2$, −0.4. Hence no significant errors are introduced by the lead remaining in solution after the usual methods of clarifying extracts with lead acetate.

Determinations with the ferricyanide reagent on solutions containing the lead salts at nine-tenths of saturation gave the following percentage errors: $PbCO_3$, +0.8; PbS, +0.4; $Pb_3(PO_4)_2$, 0.0; PbC_2O_4, +0.8. However, an appreciable effect was found (fig. 7) in the case of $PbSO_4$ which has a much higher solubility than the other lead salts
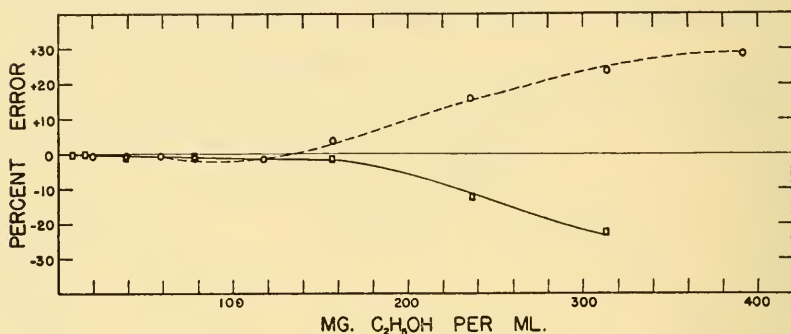

Fig. 8.—Effect of ethyl alcohol on estimation of glucose by copper (———) and ferricyanide (− − −) reagents.

investigated. For comparison the influence of normal lead acetate in the same concentration range was measured.

These results demonstrate that deleading by means of sulfate is objectionable if reducing sugars are to be estimated with the ferricyanide reagent.

Ethyl alcohol (fig. 8).—The influence of alcohol was studied in view of the possibility that it may be introduced during certain procedures for the removal of various fermentable sugars by means of micro-organisms. The results show that no significant error is caused by amounts of alcohol which might be formed from reasonably dilute sugar solutions such as would ordinarily be analyzed. The effect of alcohol on estimation of other sugars was not investigated, although it is realized, of course, that glucose would never be left after fermentation by any of the micro-organisms hitherto employed.

Table 1 has been prepared from the curves of figures 1 to 7 in order to compare the approximate minimal concentrations of various

substances which produce an error of 2 percent in the determination of 2 mg. of glucose.

Without further extension of the list of substances tested, sufficient data have been presented to demonstrate the high degree of sensitivity of these copper and ferricyanide reagents as customarily employed in analysis. It is apparent that caution should be exercised in the interpretation of results obtained from solutions containing, in addi-

TABLE 1.—*Approximate minimal concentrations of various substances causing error of 2 percent in determination of 2 mg. glucose*

Substance	Copper reagent	Ferricyanide reagent
Lead sulfate	...	0.00001 M
Lead acetate	...	.000015
Ammonium sulfate	0.003 M	...
Ammonium chloride	.006	.005
Sodium citrate	.004	.1
Magnesium chloride	.01	.003
Magnesium sulfate	.01	.003
Tripotassium phosphate	.01	.015
Calcium chloride	.015	.010
Monosodium phosphate	.025	.005
Potassium oxalate	.05	.05
Dipotassium phosphate	.06	.035
Sodium benzoate	> .1	.1
Sodium carbonate	> .175	.09
Potassium acetate	> .3	.16
Sodium chloride	.32	.25
Potassium chloride	.35	...
Sodium sulfate	.40	.22
Potassium sulfate	.40	...
Sodium fluoride	.55	.26
Sodium nitrate	.75	.27
Citric acid	.005 N	.006 N
Benzoic acid	> .016	.006
Sulfuric acid	.04	.006
Sodium hydroxide	.02	.016

tion to sugars, other components such as buffers, added reagents, or impurities of biological origin.

It should be emphasized that the results presented in figures 1 to 8 and in table 1 apply only to the specific experimental conditions (volume of reaction mixture, time of heating, nature and amount of sugar). The behavior of sugars other than glucose may differ very greatly, as illustrated in tables 2 and 3. Furthermore, the magnitude of the effect of a given substance may vary with the amount of sugar present.

These results suggest possibilities for developing reagents selective for individual sugars. Mention has already been made of the partially selective reagents for fructose devised by Fischl (1933), Strepkov (1936), and Englis and Becker (1939). A study of such reagents is in progress and will be reported separately.

TABLE 2.—*Influence of several substances on estimation of various sugars by Shaffer-Somogyi copper reagent*

| | | Percent error caused by presence in sugar solution of— | | | |
		0.05 M Sodium citrate	0.18 N Citric acid	0.25 M $(NH_4)_2SO_4$	0.3 M NaH_2PO_4
Glucose,	0.5 mg......	— 47.4	...	...	...
	1 mg......	— 37.0	...	...	...
	2 mg......	— 32.6	— 99.8	— 97.8	— 97.8
	4 mg......	— 27.8	...	...	...
	8 mg......	...	— 82.0	...	...
Fructose,	2 mg......	— 23.8	— 82.4	— 71.2	— 68.3
Maltose,	4 mg......	...	— 100.0	— 97.8	— 96.9
	5 mg......	— 1.7	...	...	...
Lactose,	5 mg......	— 4.5	...	...	...
Arabinose,	3 mg......	— 29.8	...	...	...

[1] In pure solution, approximately equal amounts of reduction are produced by: 1 mg. glucose, 1 mg. fructose, 3 mg. maltose hydrate, 3 mg. lactose hydrate, 1.5 mg. arabinose.

TABLE 3.—*Influence of several substances on estimation of various sugars by potassium ferricyanide reagent*

| | | Percent error caused by presence in sugar solution of— | | |
		0.1 M NH_4Cl	1.0 M $(NH_4)_2SO_4$	0.2 M NaH_2PO_4
Glucose,	1 mg........	— 70.5	...	...
	2 mg........	— 73.4	— 96.8	— 98.0
	4 mg........	— 75.4	...	...
Fructose,	2 mg........	— 28.2	— 75.6	— 76.3
Maltose,	3 mg........	— 79.6	...	...
Arabinose,	2 mg........	— 82.0	...	...

[1] In pure solution, approximately equal amounts of reduction are produced by: 1 mg. glucose, 1 mg. fructose, 1.5 mg. maltose hydrate, 1.2 mg. arabinose.

Introduction of a foreign substance into the reaction mixture may affect the apparent sugar oxidation in one or more of several ways: (1) it may merely interfere with the measurement of the amount of reduction; (2) it may of itself exert a reducing action on the reagent; (3) it may influence the rate of reaction between sugar and reagent; (4) it may alter the final amount of reduction.

An example of interference with the measuring technique is furnished by oxalate in the titration of ferrocyanide by ceric ion. Ow-

ing to the oxidation of the oxalate this method cannot be utilized in its presence. The interference of citrate in iodometric titration of ferricyanide and its prevention by the use of additional $ZnSO_4$ also has been mentioned. The impermanent end points observed in certain iodometric titrations in connection with the copper reagent also are probably to be ascribed to interference of this class.

None of the results described appears to be due to direct reduction of the reagent by the test substance. That a given substance of itself exerts no reducing action on a sugar reagent does not necessarily constitute evidence that the oxidation of sugar will be uninfluenced
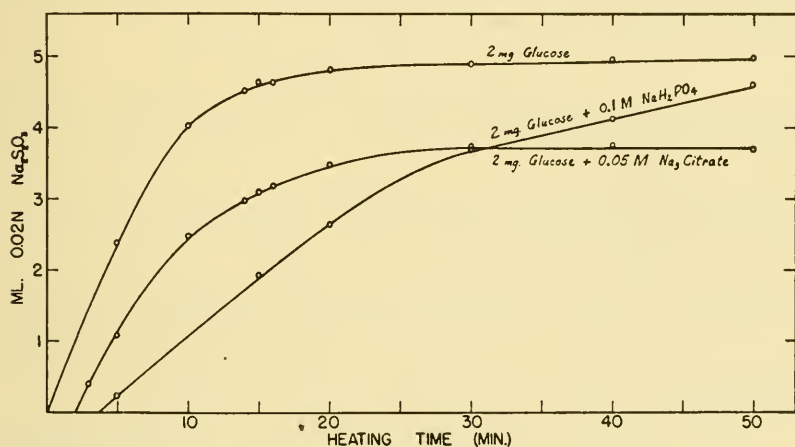

Fig. 9.—Influence of monosodium phosphate and of sodium citrate on rate of oxidation of glucose by copper reagent.

by its presence. It has been ascertained, for example, that neither of the reagents studied is appreciably reduced by substances such as citric acid, sodium citrate, sodium dihydrogen phosphate, and ammonium salts even in concentrations capable of nearly complete inhibition of the sugar oxidation under the conditions employed. Lack of appreciation of this circumstance is to be found in numerous reports in the literature of tests alleged to demonstrate noninterference by various substances.

Alterations of the rate and amount of oxidation have been discussed above in connection with the effects of hydrogen and hydroxyl ions. Further examples are illustrated in figure 9. It is apparent that the error caused by NaH_2PO_4 (or by any substance that has a similar effect) can be minimized by selecting an appropriate reaction time; this is not the case, however, for substances, such as sodium citrate, which diminish the reduction equivalent.

SUMMARY

The influence of 30 compounds, chiefly organic and inorganic acids and salts, upon the estimation of reducing sugars by copper and ferricyanide reagents has been studied. Errors are caused by the presence of most of these substances, even though of themselves nonreducing, and in some cases by concentrations which may occur naturally in solutions or extracts of biological origin or which may be introduced by certain procedures employed in carbohydrate analyses. Differences in the effects of certain of the substances upon oxidation of various sugars are so great as to suggest the possibility of devising selective reagents through their use.

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